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In Vitro Studies of the Cellbound and
Antibodymediated Immunity Evoked by
Murine Renal Allotransplants

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IN VITRO STUDIES OF THE CELLBOUND AND ANTIBODYMEDIATED
IMMUNITY EVOKED BY MURINE RENAL ALLOTRANSPLANTS

by

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INTRODUCTION

The importance of the immunological defence system against foreign antigenic agents is since long well established. The defence mechanisms are complex and can be divided into at least two main components, the T-cell system and the B-cell system (Möller, 1971).

The T-cell system is influenced by the thymus gland and is responsible for cellbound immunological responses. After stimulation of antigen receptive cells "killer cells" with specificity against the stimulating antigen are produced. The system is of importance for instance in delayed type dermal reactivity, Graft Versus Host (GVH) reactions, transplant rejection and cancer surveillance.

The B-cell system is influenced by bursa Fabricius in birds and perhaps by equivalents to this organ in mammals. The system is responsible for antibody mediated immunological responses. A helper function from the T-cell system is necessary for a normal function of the B-cell system. Usually the first antibodies to be produced after a primary antigenic stimulation belong to the Ig-M class. Later an Ig-G type of response supervenes and then inhibits the production of Ig-M antibodies by a feed back mechanism. Ig-M antibodies can be cytotoxic to antigenic mammal cells when complement is present. Ig-G antibodies likewise can be cytotoxic to mammal cells but sometimes can mediate "enhancement", that is blocking of cell surface antigens without destruction of the cells. Cytotoxic cells and/or antibodies are then prevented from reaching the target cells and thus cannot destroy them. Possibly such blocking effects are produced by antibodies in complex with free antigen rather than by antibodies alone (Sjögren & Bansal, 1971; Sjögren et al, 1971).

A serum mediated blocking effect is desirable after organ transplantation but is highly undesirable when a malignant tumor is present. The opposite holds true for cytotoxic immunological responses. Thus it is important to be able to modify the immunological response to make it more suitable in these situations. Interesting experimental results about immunological enhancement and specific immunological non-response (tolerance) have been obtained but up till now the administration of immunosuppressive drugs have been of main clinical importance (Levey 1971; Makinodan et al, 1970).

It is then naturally of major interest to investigate the mode of action of the different agents and their possible tendency for selective depres-

sion of the production of cytotoxic antibodies, cytotoxic lymphoid cells and blocking antibodies respectively. In vivo studies of the effects of different immunosuppressive agents on cellbound immunological phenomena or antibody responses have been performed extensively (References in "Discussion of the results" below). When immunosuppressive agents have been used in long time treatment after organ transplantation usually only transplant survival time, hematological data and microscopic appearance of the transplant have been recorded (Ibid). Some studies with in vitro administration of immunosuppressive agents have also been presented. Such in vitro studies give only limited information about the in vivo situation. (References in "Discussion of the results" below).

In the present study a test system is described where the effects of immunosuppressive agents in long time in vivo treatment after transplantation were investigated in the precise way possible in in vitro experiments. The test system allows selective and simultaneous study of how 1) the cellbound immunological response 2) The production of cytotoxic antibodies and 3) the production of blocking antibodies or antigen-antibody complexes were influenced by different immunosuppressive agents.

MATERIALS AND METHODS

Experimental Design

Isogenous Brown Norway (BN) rats were immunized by renal allografting using kidneys from isogenous Wistar Furth (WF) rats (Ag-B difference, Palm, 1971). The grafts were removed after five days. Control animals were otherwise unmodified and the test animals received the treatment described for the respective experimental group for 14 days. Fourteen days after the transplantation, thoracic duct lymph and blood were collected from the recipients. Recipient thoracic duct lymphoid cells and/or serum were added to cultured kidney cells of the donor strain which had been labelled with ^{51}Cr . Cytotoxic effect of the lymphoid cells and cytotoxic or blocking effects of fresh or heat-inactivated serum were reflected in the variable release of ^{51}Cr from the target cells.

Animals

Isogenous male or female WF rats weighing 150-200 g were used as transplant donors. Isogenous female BN rats weighing 200-250 g were used as recipients (Microbiological Associates, Maryland). Skin transplants interchanged within the two strains remained in place infinitely. The animals were fed on a rat pellet (Wayne lab-blox) and water diet. They were fasting during 8 hours before all surgical procedures.

Experimental Groups

Eleven groups were studied. In the different experimental groups immunosuppressive therapy was always instituted before the transplantations if this did not result in a markedly increased postoperative mortality.

- I. Control animals (10 rats). Unmodified rats received renal grafts.
- II. Presensitized animals (10 rats). These rats had carried a heterotopic WF cardiac graft (Ono & Lindsay, 1969) for 8-10 days one month prior to renal transplantation. No immunosuppressive treatment was given after either grafting.
- III. Irradiated animals (10 rats). These rats received 200 rad of total

body x-ray irradiation on days 1, 3, 6 and 10 after kidney transplantation. Each time 0.5 mm Cu and 1.0 mm Al filters and 8.8 min irradiation time were used.

- IV. Azathioprine treated animals (10 rats). These rats received azathioprine 30 mg/kg intramuscularly immediately before the transplantation and then once a day intraperitoneally.
- V. Methylprednisolone treated animals (10 rats). These rats received methylprednisolone 12 mg/kg intramuscularly just after transplantation and then once a day intraperitoneally.
- VI. ALS (p. 11) treated animals (10 rats). These rats received 0.5 ml ALS by intramuscular injection daily with the first dose given just before the renal transplantation.
- VII. Actinomycin treated animals (10 rats). These rats received actinomycin-C 50 μ g/kg intramuscularly just after transplantation and then once a day intraperitoneally.
- VIII. Cyclophosphamide treated animals (10 rats). These rats received cyclophosphamide 10 mg/kg intramuscularly immediately after transplantation and then once a day intraperitoneally.
- IX. 2 ((Methylsulfinyl) acetyl) pyridine (oxisuran) treated animals (10 rats). These rats received oxisuran 150 mg/kg intramuscularly just before transplantation and then once a day intraperitoneally.
- X. Methotrexate treated animals (10 rats). These rats received methotrexate 1.0 mg/kg intramuscularly just after transplantation and then every other day intraperitoneally.
- XI. Methotrexate and folic acid treated animals (10 rats). These rats received methotrexate like the animals in Group X but also folic acid (Leucovorin^R, Lederle) 10 mg/kg by intramuscular injection on the days between the methotrexate injections.

Surgical Procedures

Anesthesia consisted of pentobarbital sodium (Nembutal^R) 30 mg/kg intraperitoneally. When necessary it was supplemented with ether inhalation. Renal transplantation was performed using the method of Lauschke and Herman (1968) except that the ureter was left lying free in the peritoneal cavity. The recipients' own kidneys were left intact.

The thoracic duct was cannulated in its cervical portion (Saldeen & Linder, 1960). Lymph was collected in a test tube containing 0.5 ml Eagle's medium with 10 % heat-inactivated BN rat serum and PEST. The catheter (O.D. 0.024 in.) was heparinized before the operation. Before use the lymphoid cells were washed three times and centrifuged at 500 g for 10 min after each washing. The viability of the cells was ascertained by trypan blue staining.

Hematologic and Morphologic Studies

White blood cell count and differential count were performed with standard techniques. Sections of removed transplanted kidneys were studied with light microscopy after staining with Hematoxylin-eosin.

The expression "white blood cell count" is used to describe all cells, except red blood cells and platelets in blood and thoracic duct lymph. The lymphocyte count was determined by differential count among 200 white blood cells. The expression "lymphoid cells" is used synonymous with "white blood cells" when thoracic duct cells are described as leucocytes were never found in the lymph.

Antilymphocyte Serum (ALS)

Five New Zealand rabbits (weight 2-2.5 kg) were immunized intravenously three times at weekly intervals with a combination of $1-2 \times 10^8$ thoracic duct cells and $3-6 \times 10^9$ spleen and lymph node cells from a pool of out-bred Sprague-Dawley rats. Blood was collected by cardiac puncture 8 days after the last immunization and the serum was separated and pooled. Following heat-inactivation at 56°C for 30 min the serum was absorbed twice with rat red cells and sterilized by filtration. The final product had a lymphocytotoxic titer of 1/1000, a lymphagglutinin titer of 1/2000 and a hemagglutinin titer of 1/32 (Abaza & Woodruff, 1966).

Cell Cultures

Care was taken to perfuse the kidneys with serum free tissue culture medium before mincing. The kidneys were minced with a pair of scissors in a sterile Petri dish. The kidney tissue was suspended in 50 ml PBS containing 1 % foetal chicken serum and 0.05 % trypsin and was stirred magnetically for 20 min. The supernatant was then discarded and the procedure was repeated once. This second supernatant was collected and was centrifuged at 300 g for 15 min. The precipitate was suspended in

about 15 ml MEM (900 ml distilled water + 100 ml foetal calf serum, Flow 4-055 E + 100 ml Earle's solution, Flow 3-012 D + 20 ml amino acids. Flow 6-213 B + 10 ml vitamins, Flow 6-224 B + 20 ml 8 % Na HCO₃ solution + 2 ml PEST (1 million I.U. benzylpenicillin + 1 g streptomycin/20 ml H₂O)) The cell suspension was distributed among culture tubes and part of the medium was renewed every third day. When confluent monolayers had been obtained 20-23 days later, the cells were labelled with ⁵¹Cr.

At that time they contained 30-40 % epithelial cells as judged morphologically. The remaining cells were fibroblasts.

Cytotoxic Test

The WF target kidney cells were labelled whilst they were growing as monolayers in culture tubes. Sodium chromate (10-20 μ C ⁵¹Cr), (specific activity 175-300 μ C/ μ g of chromium) was then added to each tube and the cells were incubated for 15-18 hrs at 37°C. The medium was removed, the monolayers were washed three times with PBS and once with trypsin solution and were then trypsinized (0.2 % trypsin) until the cells were evenly dispersed (1.5 min + 9 min 37°C). The cells were then suspended in Eagle's medium containing 5 % foetal bovine serum. After 30 min the cell suspension was centrifuged at 300 g and the cells were again suspended in a concentration of 2×10^5 cells/ml in Eagle's medium with 5 % heat-inactivated WF rat serum. Aliquots of 0.5 ml of the cell suspension were distributed among test tubes. Various combinations of washed thoracic duct cells and fresh or heat-inactivated serum from the BN kidney recipients were added to the WF target cells in small flat bottom test tubes as shown in Table 1. The tests in experimental Group VIII were performed with slight modifications from the set up in table 1 as published earlier (Husberg 1972). Lymphoid cells and serum from a recipient rat were used within one hour after the end of collection of lymph.

The tests were done in duplicate except in Groups III and VII where this was prevented by the lack of a sufficient number of lymphoid cells in the thoracic duct lymph. The final volume in all test tubes was adjusted to 1.5 ml by the addition of Eagle's medium, containing 5 % heat-inactivated WF rat serum.

The presence of complement in the test tubes containing fresh serum and its absence in tubes without such serum were certified by use of a sheep red cell lysis system for complement titrations (Fulton & Dumbell, 1949).

At different time intervals the release of radioactivity was measured in

0.5 ml samples of the supernatants removed from each tube. 0.5 ml of Eagle's medium with 5 % heat-inactivated WF serum was added to replace the lost volume on each occasion. The radioactivity in all specimens was measured in a scintillation counter. (NUKAB - Nuclear Chicago Ultra - scaler 2 with 2x1 3/4" well type crystal or Picker Liqueumat 110 with 3 x 2 1/2" side hole crystal; 200-450 KeV; The test sample reading time was always selected so as to obtain at least 5000 counts; Test sample counts were always more than 10 times the background counts). The tubes containing packed cell deposits were repeatedly frozen and thawed before counting. The mean isotope release from each pair of duplicate tubes was used for the analysis of the results. Two calculations were required in order to obtain the total release of radioactivity into the supernatant for each studied time interval. First, the number of counts in the 0.5 ml supernatant sample had to be multiplied by 3 since the total tube volume was constantly 1.5 ml. Second, the radioactivity in previously removed samples had to be added to the findings in a subsequent sample. The amount of residual radioactivity in the cell pack at 36 hours was obtained by subtracting the radioactivity in the 36-hour supernatant, calculated as just described, from the total radioactivity in the test tube. The small error introduced by not correcting for the volume of cell pack was ignored.

The isotope release for each time interval was expressed as the per cent release of the total target cell radioactivity (Fig 4-14) and as the per cent release above the spontaneous release, "corrected release", (Brunner et al., 1968). The latter release values in the different experimental groups were compared using Student's t test when SD_1^2/SD_2^2 was less than 3.2 (Table 3). SD = standard deviation of the sample, computational

form:
$$\sqrt{\frac{N\sum X^2 - (\sum X)^2}{N^2}}$$

In all the experiments except one, the serum and the cells which were incubated together originated from rats belonging to the same experimental group. In the one exceptional experiment pooled heat-inactivated serum samples (0.3 ml) taken from recipients belonging to one of the Groups I-XI were each incubated with 10^6 thoracic duct cells from four different recipient rats belonging to Group I and 10^5 target cells (Table 3).

Kinetic Studies of the Test System

1. WF target cells and pooled Group I recipient serum were used to study the quantitative relationship between the amount of cytotoxic sera and ^{51}Cr release after 2 hours of incubation. Care was taken to keep the amount of complement in the test system at a constant level. The expe-

riment was performed four times. The findings are given in Fig. 1.

2. Target cells and pooled heat-inactivated Group I recipient serum were used to study to what extent the 12-hour blocking effect of heat-inactivated recipient serum varied with the amount of serum added. A 0.3 ml sample of heat-inactivated recipient serum was diluted in heat-inactivated serum from non-transplanted BN rats. The experiment was performed four times with thoracic duct cells from different Group I recipient rats. The results are depicted in Fig 2.

3. Thoracic duct cells from different Group I transplant recipient rats and target cells were used to study the 12-hour cytotoxic effect on target cells of different proportions of sensitized cells in the thoracic duct population. The recipient thoracic duct cells were mixed with thoracic duct cells from normal BN rats in different proportions. The experiment was performed four times and the results are shown in Fig 3.

Comparison of transplanted and nontransplanted target cells

In the different experimental groups (except II and III) the tubes 1, 3 and 6 in table 1 were also set up with target cells cultured from transplanted kidneys removed 5 days after the original surgical procedure. These tests were performed 5 times in each experimental group. This was done to test whether different immunosuppressive agents changed the sensitivity of target cells to attacks from antibodies and immunocompetent cells.

To evaluate whether invading BN host cells contaminated these cultures from transplanted WF kidneys, coverslip cultures were made from five female and five male WF kidneys which had resided in group I female BN rats for five days. After incubation at 37°C in 10 % carbon dioxide atmosphere for 10-14 days Barr body staining was performed (Klinger & Ludwig, 1957). The cultures from female transplanted kidneys and from female control fibroblast cultures contained positive peripheral nuclear Barr bodies in 25-40 % of the cells. No positive Barr bodies could be seen in cultures from male transplanted kidneys and in male control fibroblast cultures though a Barr body could not be fully excluded in 2-4 % of the cells. (It did not prove possible to stain for fluorescent Y bodies in the rat).

RESULTS

Three or less animals died within three days after transplantation in each experimental group due to technical failures. Later in the experiments, one animal in Groups II and IV, two animals in Groups VI, VII and XI, three animals in Group III and four animals in Group X were lost. New experiments replaced these mortalities.

Hematologic and Morphologic Findings

As seen in table 2 no significant differences in preoperative total white blood cell (WBC) counts or preoperative lymphocyte counts were found between the different experimental groups with the exception of the pre-sensitized group (Group II).

Before renal transplantation the mean peripheral white blood cell count in Group II was significantly higher than in the control group (Group 1). Apart from this no significant differences in blood and thoracic duct lymph cell counts were found between the two groups throughout the experiments (Table 2).

Irradiation with 800 rad in four divided doses markedly reduced the number of white blood cells/mm³ in blood and thoracic duct lymph (Table 2). The depression of thoracic duct lymph cell counts in Group III animals was significantly stronger than in any other treated group except Group VI ($p < 0.01$).

Azathioprine treated animals showed an increase in the number of white cells and lymphocytes in the blood as compared to control group animals. The number of lymphoid cells in the thoracic duct lymph was not changed (Table 2).

Methylprednisolone treatment did not cause any significant changes in peripheral blood cell counts throughout the experiments. The number of lymphoid cells in the thoracic duct lymph was decreased 14 days after the transplantations (Table 2).

Treatment with ALS markedly reduced the number of white blood cells and lymphocytes in blood and thoracic duct lymph (Table 2). At the same time, the hematocrit dropped from 50.3 ± 1.1 % to 40.2 ± 2.48 % (mean $\pm$ SD).

Fourteen days of treatment with actinomycin markedly diminished the number of white blood cells and lymphocytes in the blood. In the thoracic duct lymph the number of lymphoid cells was also clearly decreased (Table 2).

Cyclophosphamide treatment markedly diminished the total number of white cells in the blood at the time of thoracic duct cannulation (Table 2). The number of lymphocytes in blood was also diminished to a great extent. In the thoracic duct the number of lymphoid cells/mm³ was diminished to approximately 50 % in the cyclophosphamide treated group as compared to the non-immunosuppressed group.

Oxisuran therapy had no effect on the total white blood cell counts or the lymphocyte counts in blood or thoracic duct lymph (Table 2).

Methotrexate treatment markedly decreased the number of white blood cells and lymphocytes in the blood. The number of lymphoid cells in the thoracic duct lymph was also found to be diminished when tested 14 days after transplantation. When folic acid supplemented the treatment with methotrexate the decrease in the number of WBC and lymphocytes in the thoracic duct lymph was notably less (Table 2).

In all instances, 96-98 % of the thoracic duct lymphoid cells were "small lymphocytes". The flow of lymph from the thoracic duct was approximately 0.5 ml/per hour in the animals from all experimental groups except Groups III and VII. In these two groups the animals demonstrated a lymph flow averaging 0.2-0.3 ml per hour.

At the time of transplant nephrectomy the vascular anastomoses were patent in 80-90 % of the animals in all experimental groups except Group II. The kidneys showed different degrees of lymphoid cell infiltration at that time. The heaviest infiltrates appeared in kidneys from control group animals and the slightest were noted in kidneys from ALS treated animals.

In the presensitized animals (Group II) the kidneys appeared normal at transplantation. However, all of the kidneys were necrotic at the time of removal five days later.

Cytotoxicity Tests

In all experimental groups the control tubes with labelled WF kidney target cells alone or together with normal BN thoracic duct cells and normal BN heat-inactivated serum showed a 24-hour ⁵¹Cr release of

approximately 25 % (Table 1, tubes 1 & 2). Subsequent to 24 hours there was an accelerated variable spontaneous cell death (Fig. 4-14). If spontaneous 24 hours ^{51}Cr release of more than 30 % was obtained in a single experiment all the results were discarded. The 36 hr results are not presented in Table 3 as they were inconclusive due to large standard deviations.

Group I. Control Animals. Thoracic duct lymphoid cells from BN recipient rats had a clear cytotoxic effect on WF kidney target cells after 12 hours of incubation (Fig. 4, Table 3). After only two hours a moderately strong cytoaggressive effect of the fresh serum from recipient rats was noted. Further incubation caused no major increase in isotope release from target cells as compared to the spontaneous release. The cytotoxic effect of sensitized lymphoid cells was significantly reduced (27.3 as compared to 36.2 per cent "corrected" release at 12 hr, $p < 0.001$) when heat-inactivated serum from the BN recipient rat was added 15 min before the lymphoid cells.

Group II. Presensitized Animals. Thoracic duct lymphoid cells, as well as fresh serum from recipient rats, had an increased cytoaggressive effect compared with the findings in the control group (Fig. 5, Table 3). The cytotoxic effect of lymphoid cells together with heat-inactivated serum from these rats was not significantly different from that in the non-immunosuppressed control group indicating an increased blocking capacity of the heat-inactivated sera from the presensitized recipients.

Similarly, in the experiment where heat-inactivated pooled recipient serum from either presensitized or control animals was added to the target cells prior to standard samples of sensitized lymphoid cells, the ^{51}Cr release was considerably lower with the first serum (Table 3). Using the quantitative relationship established in the kinetic experiments, this difference in release corresponded to a 2-2.5 fold increase in the amount of blocking serum (Fig. 2).

Group III. Irradiated Animals. Thoracic duct lymphoid cells from these animals caused an isotope release similar to that in control animal experiments (Fig. 6, Table 3). The cytotoxic effect of fresh recipient serum on target cells was markedly reduced, corresponding to a 80-85 % decrease in the amount of cytotoxic antibodies according to the relationship established in the kinetic experiments (Fig. 1).

In the experiment where heat-inactivated pooled recipient serum from

either the irradiated or the control group was added to the target cells prior to standard samples of sensitized lymphoid cells, the blocking effect was considerably less when the first serum was used (Table 3). This indicated a diminished blocking activity of heat-inactivated recipient serum. The change corresponded to a 80-90 % decrease in the amount of blocking serum (Fig. 2). The cytotoxic and blocking effects of Group III sera were significantly more depressed as compared to sera from any other treated experimental group ($p < 0.01$).

Group IV. Azathioprine treated Animals. Thoracic duct lymphoid cells from these animals did not have a different cytotoxic effect on target cells as compared to those from control animals (Fig. 7, Table 3). The cytotoxic effect of fresh recipient serum was moderately reduced (approximately 25 % decrease in the amount of cytotoxic antibodies as judged by the dilution experiments performed earlier). A small but still significant blocking effect (29.0 as compared to 36.4 per cent "corrected release" at 12 hr, $p < 0.001$) of heat-inactivated recipient serum on cell-mediated cytotoxicity also occurred in Group IV. This was investigated further in the additional experiment where heat-inactivated pooled recipient serum from azathioprine treated and control group animals were separately added to the target cells prior to standard samples of sensitized thoracic duct cells. As judged by the dilution experiments the difference in blocking effect as compared to Group I sera corresponded to a 20-30 % decrease in the amount of blocking serum that was present. The difference was however not significant (Table 3).

Group V. Methylprednisolone treated Animals. When recipient thoracic duct lymphoid cells were tested there was a clearly diminished isotope release from target cells as compared to the results in the control group (Fig. 8, Table 3). There was also a considerable reduction in the cytotoxic effect of complement containing recipient serum. The changes corresponded to an approximate 25 % decrease in the number of sensitized lymphoid cells (Fig. 3) and to a 45-50 % decrease in the amount of cytotoxic antibodies in the dilution experiments (Fig. 1). The blocking effect of heat-inactivated serum was moderately reduced as shown in the additional experiment (Table 3) where heat-inactivated pooled recipient serum from methylprednisolone treated and control group animals were compared in an identical target cell - lymphoid cell system (corresponding to approximately 30 % decrease in the amount of blocking antibodies or complexes in serum, Fig. 2).

Group VI. ALS treated Animals. When recipient lymphoid cells were added

to target cells there was a markedly diminished isotope release compared to the control group results (Fig. 9, Table 3). The change corresponded to an approximate 50 % decrease in the number of sensitized thoracic duct lymphoid cells that was present. The cytotoxic effect of complement containing recipient serum on target cells was moderately reduced (about a 25 % decrease in the amount of cytotoxic antibodies according to the earlier dilution experiments). In the additional experiment where heat-inactivated pooled recipient sera from the ALS treated and the control group were compared under standardized conditions, the blocking activity of serum from ALS treated recipients was found to be smaller. The change corresponded to an approximate 30 % decrease in the amount of blocking serum that was present (Fig. 2).

Group VII. Actinomycin treated Animals. Compared to the control group results the isotope release from target cells was not diminished when recipient thoracic duct cells from Group VII were used (Fig. 10, Table 3). On the contrary a small increase in the mean cytoaggressive effect per 10^6 thoracic duct cells was seen at one time interval, but because of the higher standard deviation ($SD_1^2/SD_2^2 = 4.1$) in the actinomycin treated group where the tests were not performed in duplicate a reliable t-value for homogenous variance could not be obtained. The cytoaggressive effect of complement containing recipient serum was moderately reduced also in this group (corresponding to an approximate 25 % decrease in the amount of cytotoxic antibodies that was present). A blocking effect of heat-inactivated recipient serum on lymphoid cell mediated cytotoxicity was found also in this group. In the additional experiment, comparing Group I and Group VII pooled heat-inactivated recipient sera this blocking effect was smaller when serum from the actinomycin treated group was used (corresponding to a 30-40 % decrease in the amount of heat-inactivated recipient serum that was present).

Group VIII. Cyclophosphamide treated Animals. The percentage isotope release is shown in Fig. 11 and the percentage "corrected" isotope release is shown in Table 3. The corresponding control group results are presented earlier (Husberg, 1972). When recipient thoracic duct cells was tested there was a small but significant ($p < 0.01$) difference in isotope release as compared to the non-immunosuppressed group. The inhibitory effect of heat-inactivated recipient serum was similar to that seen in the non-immunosuppressed group. The initial cytoaggressive effect of recipient serum with complement was, however, significantly reduced.

Group IX. Oxisuran treated Animals. Recipient thoracic duct cells from

Group IX animals caused a diminished isotope release from the target cells as compared with those from Group I rats (Fig. 12 and Table 3). According to kinetic studies performed the change corresponded to a 15 to 20 % decrease in the number of sensitized thoracic duct cells that were present. The cytotoxic effect of fresh, complement containing recipient serum was not altered significantly by oxisuran therapy.

In the exceptional experiment in which pooled heat-inactivated recipient serum from Group I or Group IX rats was added to the target cells before the introduction of standard samples of sensitized thoracic duct cells the blocking effect was similar when either serum was used (Table 3). This indicates that the blocking effect of heat inactivated recipient serum was not altered by oxisuran therapy.

Group X. Methotrexate treated Animals. When recipient thoracic duct lymphoid cells were tested there was a clearly diminished isotope release from target cells as compared to the findings in the control group (Fig. 13, Table 3). There was also a considerable reduction in the cytotoxic effect of complement containing recipient serum. Using the quantitative relationship established in the dilution experiments the changes corresponded to an approximate 50 % decrease in the number of sensitized lymphoid cells and to a 40-45 % decrease in the amount of cytotoxic antibodies. A doubtful (12.0 as compared to 15.0 per cent "corrected release" at 12 hr, $p < 0.05$) blocking effect of heat-inactivated recipient serum on cell-mediated cytotoxicity also occurred in Group X. In the experiments where heat-inactivated pooled recipient serum either from the methotrexate treated or the control group was separately added to the same target cells prior to standard samples of sensitized thoracic duct cells, the blocking effect was markedly smaller when the former serum was used. This indicated a diminished blocking activity of heat-inactivated recipient serum. The change corresponded to a 40-50 % decrease in the amount of blocking serum that was present.

Group XI, Methotrexate and Folinic Acid treated Animals. Also in this group thoracic duct lymphoid cells as well as fresh serum from BN recipient rats had a decreased cytoaggressive effect as compared to the control group results (Fig. 14, Table 3). The obtained isotope release values were not significantly different from the results of the corresponding experiments in the Group X animals. According to the earlier kinetic studies of the test system, the decrease corresponded to an approximate 45 % decrease in the number of sensitized lymphoid cells and to a 35-40 % decrease in the amount of cytotoxic antibodies. The blocking effect of heat-inactivated recipient serum was moderately reduced as could also

bee seen in the additional experiment (Table 3) where heat-inactivated pooled recipient serum from either the methotrexate-folinic acid treated or the control group was added to the target cells prior to standard samples of thoracic duct lymphoid cells (Approximately 30 % decrease in the amount of blocking antibodies or complexes in serum).

Group I-XI. In all groups, when fresh recipient serum and lymphoid cells were added to the target cells in combination, the early isotope release pattern was similar to that with fresh serum alone, while after 12 hours the release curves resembled those obtained with lymphoid cells alone. (Fig. 4-12, line 5; table 3).

Likewise in all experimental groups, the addition of further complement-containing serum from normal BN rats (Table 1, Tubes 7 & 8) did not significantly change the cytotoxic effects caused by fresh recipient serum or recipient lymphoid cells. This confirmed that the need for complement in the tubes with fresh recipient serum was satisfied and that the cell-mediated cytotoxicity was independent of complement.

No significant difference was found in any experimental group when cells from transplanted and non-transplanted kidneys respectively were used as target cells.

DISCUSSION OF THE EXPERIMENTAL MODEL

Many authors have reported that lymphoid cells and/or serum from animals or human beings sensitized against target cell antigens have a cytotoxic effect on these target cells. Different allogeneic and xenogeneic systems have been used but mostly these have not involved the sensitive ^{51}Cr release assay. Most experiments hitherto performed have been systematically tabulated and analysed by Dutton (1967) and by Perlmann & Holm (1969).

Serum has sometimes been found to exert a cytotoxic effect in the presence of complement (Kalfayan & Kidd, 1953; Stetson & Jensen, 1960; Terasaki et al, 1961; Koprowski & Fernandes, 1962; Möller, 1965b; Holm, 1967; Hellström et al., 1968a, b). In other experiments it has been found to have no cytoaggressive effect or to block the cytoaggressive effects of lymphoid cells (Brunner, Mauel & Schindler, 1967; Rosenau, 1963; Möller, 1965b; Wilson, 1965; Holm, 1967; Brunner et al., 1968). In still other experiments it has been reported that a cytoaggressive effect on target cells was only obtained when serum and lymphoid cells from sensitized animals were present (Govaerts, 1960; Holm 1967; Bubenik, Perlmann & Hasek, 1970). In experiments where no cytotoxic activity has been found in serum, immunization has mostly been made by the subcutaneous or intraperitoneal route or by a skin transplant still in place at the time of the in vitro cytotoxic experiment.

Use of ^{51}Cr release for evaluation of cytotoxic effect in vitro is reported previously (Goodman, 1961; Sanderson, 1964; Wigzell, 1965; Holm, 1967; Ankerst & Sjögren, 1969). The assay is simple and sensitive and released ^{51}Cr is not reutilized by surviving cells. However, it has a high spontaneous release of radioactivity. The assay is discussed thoroughly by Perlmann & Holm, (1969). Compared to the results reported here other authors have reported clearly more rapid cytoaggressive effects of lymphoid cells on target cells but a higher ratio lymphoid cells/target cells has then been used, mostly around 100/1. That proportion then was chosen according to a "single hit" theory between lymphoid cells and target cells with an estimated proportion of 1 % specifically sensitized cells in the population of lymphoid cells (Wilson, 1965; Brunner et al., 1968; Perlmann & Holm, 1969).

In the experiments reported here thoracic duct lymphoid cells were used for obtaining a homogeneous (95 %) population of small lymphocytes and a population of recirculating thymus derived cells known to mediate cell

bound immunity (Möller, 1971). Thoracic duct cells are known to be able to show a cytoaggressive activity in vivo and in vitro (Gowans, McGregor & Cowen, 1962; Berg & Källen, 1963; Billingham, Silvers & Wilson, 1963; Wilson 1963, 1965; Brunner et al, 1970).

The differences in cytotoxic activity were always evaluated by the amplitudes of isotope release in corresponding experiments in the different groups of rats. In the tests concerning serum effects such differences could also have been evaluated by serial dilution of the sera and determination of a dilution titer capable of exerting a certain cytotoxic effect. Such a test system could be less sensitive to unspecific variations in the isotope release causing large standard deviations within some groups of results presented here. Also, several tests could then have been performed on one occasion with one batch of target cells. However in the experiments dealing with the cell mediated cytotoxic effects a dilution type test system is difficult to use. It is however important to obtain results of both cellbound and antibodymediated immunological events in a comparable way and therefore a test system measuring the amplitudes of isotope release was chosen. The duplication of all test tubes diminished the influence of single erroneous tests on the standard deviations within the different groups of results. The test system also allowed study of a single test tube repeatedly at different time intervals. In one experiment it was also possible to compare the blocking activity of different sera using the same target cells and the same thoracic duct cells.

The time interval of 14 days between renal transplantation and collection of cells and serum for cytotoxicity tests was chosen in order to obtain optimal amounts of both cytotoxic and blocking activity in the recipient serum. The transplant was removed 9 days before the in vitro experiments. This prevented any possible absorption of serum antibodies to the transplant and should allow the serum levels of specific antibodies to rise. In preliminary experiments it could be shown that the cytotoxic activity in serum was higher if the recipient animals were bled earlier after transplant nephrectomy. At this time, however, the blocking activity in the serum was considerably less. The cytotoxic effect of recipient serum could also be increased by the addition of guinea pig complement to the test system but the possibility of non-specific cytotoxicity from the xeno-geneic proteins made such an addition undesirable (Möller, 1965a; Levy 1958). The long time interval which was allowed to elapse between renal transplantation and the sampling of sera, and the use of rat complement might thus explain why such large amounts of fresh recipient serum were required to evoke the cytotoxic effects on target cells in this test system. It is possible that rat complement or even complement from certain rat strains has an inferior capacity to bind to antigen-antibody complexes. Evidence for such a phenomenon has recently been presented (French,

1972). The fact that the cytotoxicity of fresh recipient serum was not enhanced by adding further fresh normal rat serum proves, however, that rat complement was present in sufficient quantities in the pertinent tests.

In all experimental groups, each recipient serum sample invariably displayed a cytotoxic effect in the presence of complement and a blocking effect in its absence. Such dual effects of a single serum specimen have been reported earlier by French and Batchelor (1969) who found that sera from rats sensitized to transplant donor antigens were cytotoxic to donor lymphocytes in vitro when guinea pig complement was present but gave an in vivo prolongation of transplant survival by enhancement. In the present test system the phenomenon can be explained by serum antibodies which were cytotoxic in the presence of complement but blocked target cell antigen determinants when complement was absent. Alternatively, formation of blocking antigen-antibody complexes in the incubation medium could occur (Sjögren & Bansal, 1971; Sjögren et al., 1971). Finally, there is the possibility of the presence of two kinds of antibodies which competed at the antigen determinants of the target cells. In this case one would have to speculate that the blocking antibodies could not fully prevent the effect of the cytotoxic antibodies in vitro when rat complement was present. Such a simultaneous presence of different kinds of antibodies have been demonstrated by Mullen & Hildeman (1971) who found that the Ig-G fraction of sera from rats sensitized to transplant donor antigen prolonged skin transplant survival in the new host whereas the Ig-M fraction diminished the survival time. The situation in the test system described here can only be clarified by fractionation of recipient sera.

The absence of an additive effect when recipient lymphoid cells and complement containing recipient serum were added together to target cells in the experiments reported here might depend on the direction by chemotaxis of lymphoid cells to target cells having already been damaged by cytoaggressive antibodies. A similar absence of additive effect when sensitized lymphoid cells and complement containing recipient serum were added to human transplanted kidney target cells has been reported by others (Wolf et al., 1971).

Perlmann and others have reported that antigenic target cells, treated with certain heat-inactivated antisera, were damaged when exposed to a moderate excess of lymphoid cells from unsensitized donors. The antisera were effective at dilutions too high to cause conventional complement-mediated lysis in the absence of lymphoid cells. Xenogeneic, allogeneic and autogeneic systems could be used. (For references and discussion see Perlmann & Holm, 1969). In the experiments reported here a similar effect might sometimes have been expected. No additive

effect was, however, obtained when recipient rat lymphoid cells and recipient rat serum were added to target cells together, though the results obtained with complement present were rather variable ($SD \pm 2-8 \%$). The differences between the results may perhaps be explained by differences in dose levels, since it was found in the experiments by Perlmann and others that high concentrations of antiserum were inhibitory. Furthermore, ratios of 25/1-50/1 between lymphoid cells and target cells were found optimal. The antibodies inducing cell-mediated cytoaggressivity were predominantly of 7S-type, and no correlation was found between the cytoaggressive titre of an antiserum and its cytoaggressive potency in the cell-mediated test.

DISCUSSION OF THE RESULTS

The proportions of sera, lymphoid cells and target cells in the test system was chosen so as to obtain ^{51}Cr release values in the control group experiments (Group I) suitable for comparison with those in the experimental groups II-XI.

Rats in Group II having rejected a kidney in a second set fashion had thoracic duct lymphoid cells with an augmented cytotoxic activity. This suggests an increased proportion of sensitized cells in the thoracic duct lymphoid population. The uncorrected release of 55 % of the radioactivity in 12 hours in this experiment indicates that the great majority of the target cells was killed during this time (70-75 % uncorrected ^{51}Cr release corresponds to the death of all target cells). It is of interest that both the cytotoxic and blocking effect in serum were increased in animals which had undergone two consecutive transplants.

Among the irradiated animals (Group III) a depression of cell-bound immunity was expressed by a marked decrease in the number of circulating lymphoid cells in the thoracic duct lymph but not by a decreased cytotoxic capacity among the remaining cells. Thus, sublethal irradiation in divided doses prevented production of normal amounts of small recirculating lymphocytes but the lymphoid cell population surviving or produced in spite of the irradiation had an intact ability to attack target cells. Irradiation markedly decreased both blocking and cytotoxic activity of recipient serum probably by affecting antibody producing cell-lines. No tendency to selective depression of blocking or cytotoxic effect in serum, respectively, could be noted.

Although total body irradiation appears to be more effective in suppressing an antibody response to an antigen, it has also been shown to suppress cell-mediated immune responses in several species (Uhr & Scharff, 1960; Brooke, 1962; Bowser, Spruce & Exum, 1970; Visakorpi, 1970) and prolong transplant survival (Brooke, 1962; Hamburger et al., 1962). The antibody response is most sensitive to irradiation during its induction phase and is less sensitive to irradiation during the phase of antibody production (Dixon, Talmage & Maurer, 1952). The secondary response is also less easily depressed (Silverman & Chin, 1954; Tyan & Cole, 1963). Thus, the time relationship between introduction of antigens and irradiation is important (Brooke, 1962) and at some time intervals even enhancement of antibody response can be obtained (Dixon & McConahey, 1963; Stoner & Terres, 1963).

Irradiation thus has a complex "multitarget" effect on the immune response, depressing the early stage of antigen handling in macrophages, as well as late stages of the immune response (Gallily & Feldman, 1967; Jansen et al., 1969). The 7S and 19S response has been reported to be depressed to different extents by irradiation (Svehag & Mandel, 1964; Santos, 1967; Nettesheim, Williams & Hammons, 1969). Memory cells inactivated by irradiation cannot recover spontaneously but constitution of a new memory cell pool can take place later if the antigen is still available at that time (Nettesheim & Williams, 1968).

Azathioprine treatment (Group IV) caused no change in cell-bound immunological events after kidney transplantation; that is, in Group IV animals the number of lymphoid cells in the thoracic duct lymph and the cytotoxic activity of the lymphoid cells was unchanged as compared to control group animals. The amounts of cytotoxic and possibly blocking activity in serum were moderately decreased in azathioprine treated animals. The increase in peripheral white blood cell and lymphocyte counts during azathioprine treatment is unexplained. Similar reactions can sometimes be noted when the drug is used in dog experiments and clinically.

Azathioprine is to a great extent degraded to 6-mercaptopurine in vivo (Elion et al., 1961). Both agents are good immunosuppressive and antiflogistic agents with a well known mode of action in several species including man (Starzl et al., 1964b; Starzl et al., 1965; Elion, 1967). However, they have often been reported to be ineffective or doubtfully effective in protecting transplants in rats and mice, suggesting failure to suppress the cell-bound immunological defense in these species. Evidence for such lack of effectiveness after rat kidney transplantation has been published by Shehadeh et al. (1970) and for skin transplants by Hubay, Powell & Holden (1960) and Santos & Owens (1965). Furthermore, in murine GVH systems which involve cell-mediated immune reactions, azathioprine can increase mortality (Schwartz & Beldotti, 1965). In contrast to these findings, significant prolongations of rat kidney transplants with comparatively low doses of azathioprine have been reported by Tinbergen (1968). Sensitized rat lymphocytes also have an inhibited capacity to attack target cells in vitro if azathioprine is present in the incubation medium in doses which do not kill the lymphocytes (Wilson, 1967).

Antibody responses to many antigens, on the other hand, can be suppressed by purine analogues also in rats and mice (Frish & Davies, 1962; Santos & Owens, 1964; Haines, Johnson & Petering, 1967). In the rabbit, 6-mercaptopurine has been reported to inhibit preferentially 7S antibody

response to an antigen leaving the 19 S antibody response relatively undisturbed (Sahiar & Schwartz, 1964). If, in the present experiment the blocking effect of recipient serum had been more depressed than the cytotoxic effect, this could have been compatible with these earlier results, such a discrepancy, however, was not found. Also in the rabbit, 6-mercaptopurine can even enhance antibody response to an antigen under certain circumstances (Chanmougan & Schwartz, 1966). Single high dose treatment with azathioprine appears to be more effective to suppress an antibody titer than repeated low doses (Berenbaum, 1967).

Treatment with a corticosteroid agent (Group V) depressed cell-bound immunological responses. This depression was shown both by a decrease in the number of cells in the thoracic duct lymph and by a decreased cytotoxic capacity among the remaining cells. The cytotoxic effect of Group V recipient serum was diminished relatively more than the blocking effect. This could indicate a preferential depression of 19 S antibody response in methylprednisolone treated animals. 19 S antibody response to sheep red cells as measured in Jerne plaque assay can be inhibited by corticosteroids in vitro (Strander, 1966). On the other hand, selective depression of the *in vivo* 7 S response has been noticed in sheep red cell-mice systems (Petrányi et al 1971).

Corticosteroids affect the immunological response in several ways in all species tested. A suppression of antigen handling in macrophages might be an important mode of action (Lurie et al., 1952; Dukor & Dietrich, 1968) as it is known that with moderate doses of corticosteroids the antibody response to many antigens can only be effectively inhibited during a short time interval close to the administration of antigen (Berglund, 1965). With high doses, antibody-mediated primary responses and to a lesser degree secondary responses can also be depressed during ongoing antibody production in many species (Fischel, Vaughan Photopoulos, 1952; Fagraeus & Berglund, 1961). This depression may partly be caused by a decrease in protein synthesis (Woll & Weinschelbaum, 1959).

The cell-bound immunological events are depressed partly by the anti-inflammatory action of corticosteroids stabilizing the cell membranes (Review by de Duve, 1964). Also, sensitized lymphocytes are killed or prevented from expressing cytotoxic effects by corticosteroids (Dougharty, Berliner & Berliner, 1960). Corticosteroids have a well documented depressive effect on delayed hypersensitivity phenomena (Gell & Hinde, 1951; Osgood & Favour, 1951) and on processes considered to involve cell-mediated immunological responses; such as, experimental allergic encephalomyelitis (EAE) (Gammon & Dilworth, 1953), GVH reactions (Schwartz & Beldotti, 1965), and rejection of transplants (Billingham, Krohn & Medawar, 1951; Medawar & Sparrow, 1956; Starzl et al, 1964a). Finally, corticosteroids can influence the complement system in in vitro studies al-

though in vivo studies have been inconclusive (Review by Gewurz et al., 1965).

In the ALS treated animals (Group VI) the decrease in number of cells in thoracic duct lymph and the decrease in cytotoxic capacity of the remaining cells indicate a marked suppression of cell-bound immunological responses to the transplants. The reduced cytotoxicity could be caused by a decreased proportion of sensitized lymphoid cells or by a partial inactivation of all lymphoid cells diminishing their ability to attack target cells. Furthermore antilymphocytic antibodies blocking the cytotoxic effect of thoracic duct cells could be partially removed when the thoracic duct cells were washed before the in vitro tests. It is therefore possible that the thoracic duct cells were even less cytotoxic to target cells in vivo. It is interesting that the number of "small lymphocytes" was diminished to a greater extent in thoracic duct lymph than in blood. This supports earlier investigations suggesting that ALS mainly affects the recirculating pool of thymus-derived lymphoid cells responsible for expression of cell-bound immunity (Review by Lance, 1970). ALS treatment also moderately decreased both cytotoxic and blocking activity of recipient serum. This is probably caused by a depressive effect on antibody producing cell lines, perhaps by way of suppressing thymus-derived "helper cells". The results reported here thus suggest that ALS mainly depresses cell-bound immunological responses. This is in accordance with earlier investigations (Review by Sell, 1969).

In the actinomycin treated group (Group VII), cell-bound immunity was depressed by a decrease in the number of cells in the thoracic duct lymph but not by a decreased cytoaggressive capacity among the remaining cells. The lymph flow in the thoracic duct was also diminished 14 days after transplantation. The cytotoxic and blocking activity in serum was decreased in recipient animals from this group.

Actinomycin-C, and the closely related compound actinomycin-D, (Brockman, 1960) are thought to bind the guanine residue of DNA thereby inhibiting DNA directed RNA synthesis by making the DNA inaccessible to RNA polymerase (Goldberg & Reich, 1964). Their influence on cell-bound immunological defense in vitro seems to be weak (David, 1965). A weak prolongation of skin-transplant survival in rats using borderline toxic doses of actinomycin-C has been reported by Busse, Boese & Bucherl (1968). Actinomycin-C and -D in vivo depresses the antibody response to an antigen in many species (Hadnagy et al., 1959; Nathan et al., 1961) and also in the rat (Wust, Gall & Novelli, 1964). In rat cell in vitro systems, a depressive effect of actinomycin-D on secondary antibody response has also been reported (Mitchell, 1964). Fishman, van Rood & Adler (1965) used a rat cell in vitro system where antigen was

incubated with macrophages together with actinomycin-D. The disrupted macrophages were then transferred to rat lymph node cell cultures. A decrease of the early 19 S antibody response was seen but the 7 S antibody response was not diminished. A finding in the experiments reported here that the cytotoxic effect of complement-containing recipient serum was depressed more than the blocking effect of heat-inactivated recipient serum could have been equivalent to the findings of Fishman et al. but such a pattern was not seen.

In the cyclophosphamide treated group (Group VIII), cell bound immunity was depressed by a decrease in the number of cells in the thoracic duct but only to a small degree by a decreased cytoaggressive capacity among the remaining cells. The cytotoxic effect of Group VIII recipient serum appeared to be diminished more than the blocking effect. This might indicate a selective depression of the 19 S response.

Cyclophosphamide belongs to the group of immunosuppressive drugs called alkylating agents. It is converted to its active form in the liver (Foley, Friedman & Drolet, 1961, Brock et al., 1971) and appears to be effective in suppressing immune responses in man and in rodents, especially guinea pigs (Maibach & Maguire, 1963; Starzl et al. 1971, Gabrielsen & Good, 1967). However, it is doubtfully efficient in the dog (McPhee & Wright, 1964; Starzl et al., 1972, unpublished data). Small repeated doses of cyclophosphamide have been found to be more effective than single massive doses (Potel, 1965).

Cyclophosphamide is known to markedly suppress antibody responses to antigens and is thought by many to suppress preferably bone marrow derived "B cells". (Staples & Talal, 1970; Linna et al., 1972; Nirmul & Taub, 1972; Turk & Poulter, 1972). 7 S and 19 S responses are depressed to different extents when the treatment schedules are varied (Santos & Owens, 1966). However, it does also diminish delayed type hypersensitivity, although this is tested mainly in guinea pigs and the hypersensitivity state can reappear when the number of white blood cells rises after the treatment is discontinued (Maguire & Maibach, 1961; Turk & Stone, 1963; Turk, 1964). Cyclophosphamide also influences other reactions thought to involve cellbound immunological responses such as EAE (Calne & Leibowitz, 1963) and rejections of allogeneic transplants (Santos and Owens 1965; Starzl et al., 1971).

Quite recently the effect of a single in vivo dose of cyclophosphamide on the in vitro cytotoxicity of mouse spleen cells has been studied with ^{51}Cr technique (Fass and Fefer, 1972). The mice were sensitized to tumor cells. It was found that a large dose of cyclophosphamide administered in vivo after the antigen decreased the in vitro cytotoxicity per given num-

ber of spleen cells to target tumor cells. On the other hand cyclophosphamide given before the antigen increased the cytotoxicity.

The animals treated with oxisuran showed a diminished cellular immune response to the kidney allografts. This was not caused by a decrease in the number of lymphoid cells in thoracic duct lymph but by a decreased cytotoxic effect of the cells. This may have resulted from a decreased proportion of sensitized lymphoid cells or by a partial inactivation of all lymphoid cells diminishing their ability to attack target cells.

Oxisuran therapy did not alter the cytotoxic effect of fresh recipient serum indicating that the antibody producing cells and their precursors were left undisturbed by this treatment. The results of the kidney transplant experiments, which showed a selective depression of cellular immunity, are in keeping with findings in heart transplant studies, in which moderate prolongation of graft survival occurred with oxisuran therapy (Husberg & Penn, 1973).

Oxisuran, 2 ((methylsulfinyl)acetyl) pyridine is a new immunosuppressive agent, which is claimed to have the unique property of inhibiting cell-mediated immunity without any concomitant effect on humoral antibody formation (Freedman et al., 1972). Its mechanism of action is not yet known. It is claimed not to be cytotoxic nor antiproliferative and it does not exhibit antiinflammatory activities (Freedman et al, 1972) Previous studies have shown that it can prolong skin allograft survival in mice, rats, rabbits and dogs (H. H. Freedman, personal communication 1972).

Treatment with methotrexate depressed the cell-bound immunological responses. This depression was shown both by a decrease in the number of cells in the thoracic duct lymph and by a decreased cytotoxic effect among the remaining cells. The cytotoxic and blocking effects of fresh and heat-inactivated recipient sera were both markedly diminished. A tendency toward selective suppression of the blocking activity of recipient sera could have been compatible with earlier experimental data showing that methotrexate primarily depresses 7 S antibody responses (Blinkoff, 1966; Santos & Owens, 1966). However, such a selective depressive effect was not found in the experiments reported here.

When folic acid was added to the test system the depression of the lymphoid cell amount in the thoracic duct lymph was markedly less. However, the cytotoxic effect per 10^6 lymphoid cells was essentially unchanged as compared to the results of the experiments with methotrexate treatment only. Likewise, the cytotoxic and blocking effects of recipient serum were not changed significantly.

Methotrexate is an effective immunosuppressive agent in many species

(Bertino, Hillcoat & Johns, 1967). It works by inhibition of the enzyme, dihydrofolate reductase, thus preventing the conversion of folic acid to tetrahydrofolic acid (Bertino et al., 1967). Folic acid antagonists suppress both delayed type hypersensitivity (Pritchard & Hayes, 1961; Turk & Stone, 1963; Mitchell et al., 1969) and antibody-mediated immunity (Malmgren, Bennison & McKinley, 1952; Santos & Owens, 1962). They can also prolong skin transplant survival (Hechtman et al., 1962; Santos & Owens, 1965) and depress GVH reactions (Schwartz & Beldotti, 1965). Higher total doses can be tolerated if the agent is administered with longer time intervals between consecutive doses (Berenbaum, 1967).

It has been shown in mice that methotrexate only requires 6-8 hours to complete its action on the immune response to an antigen. Thus, mice given a dose of folinic acid 8 hours after a single dose of methotrexate gained considerable protection against weight loss and death. Still they showed a marked depression of antibody response to the antigen (Berenbaum & Brown, 1965). This principle has been used to increase the therapeutic efficiency of methotrexate in mice leukemia treatment (Mead et al., 1963) human leukemia treatment (Mitchell et al., 1969) and in immunosuppression for allogeneic guinea pig skin transplants (Berenbaum, 1964).

No differences in sensitivity to attacks was found between transplanted and non transplanted target cells throughout the experiments. However, such a comparison between transplanted and non-transplanted target cells is complicated by two factors: First, cells of recipient origin could invade the transplant from the blood stream and could then possibly grow in the monolayers of cells cultured from the transplants. They would then disturb the quantitative relationship between target cells and lymphoid cells by not taking part in the cytotoxic reaction. This possibility is made highly unlikely by the Barr body staining experiments. Second, it is possible that the proportions of fibroblasts and epithelial cells were different in the cultures from transplanted and non-transplanted kidneys. As fibroblasts might be less sensitive to attacks than epithelial cells (Govaert, 1960) a possible change in sensitivity to attack caused by an immunosuppressive agent could be masked by a changed proportion of fibroblasts in the cultures from the transplanted kidneys. However, as no differences between non-transplanted and transplanted target cells was found in the control group without immunosuppression, such differences in cell composition of the cultures appear to be unimportant. Thus it can be stated that the tested immunosuppressive drugs are unlikely to cause any changes in target cell sensitivity to attacks.

COMPARISON OF DIFFERENT IMMUNOSUPPRESSIVE AGENTS

Single high dose treatment with some immunosuppressive drugs appears to be more effective to suppress an antibody response than repeated low doses (Berenbaum, 1967) but was not used in these experiments designed to investigate the rejection of allogeneic kidney transplants as close as possible to the clinical situation.

As pointed out by Santos (1967) immunosuppressive agents, when compared, should be used in "equitoxic doses". In the experiments reported here a comparison between the different immunosuppressive agents in true "equitoxic" doses could only have been performed if reliable LD₅₀ toxicity doses for this very test system had been available for all immunosuppressive agents. This would necessitate hundreds of transplantations and treatment courses. Also, a chosen treatment schedule in a certain experimental group might then have killed all rats if it had been continued slightly beyond 14 days, while in another experimental group the selected dose might have been tolerable for a long time. Thus the results of comparisons between immunosuppressive agents used in doses so obtained would not give quite satisfactory knowledge of their general efficiency in long time treatment as compared to each other. In the experiments reported here the doses administered were therefore always chosen as closely as possible to known maximal tolerated doses for long time treatment in the rat (Santos & Owens, 1964; Berenbaum, 1967; Santos, 1967; Busse et al, 1968; Eisele & Bücherl, 1969; Makinodan et al, 1970; Data from manufacturers). When a certain treatment schedule then gave an unacceptable mortality in this test system involving two intraabdominal surgical procedures, a somewhat lower dose had to be chosen for that experimental group. When adequate toxicity information was not available for a certain treatment group (for instance group VI) 10-15 pilot experiments had to be performed to obtain such information.

Still the results of the investigations reported here do not allow comparisons between minor variations in the immunosuppressive efficiency of different agents. Instead data about the mode of action of different immunosuppressive agents and major differences in immunosuppressive efficiency are obtained.

In this test system total body irradiation had a superior depressive effect on cytotoxic and blocking activity in recipient serum as compared to the investigated immunosuppressive drugs (table 3). Methotrexate likewise had a marked depressive effect on antibody mediated immunological re-

sponses. Cyclophosphamide and methylprednisolone treatment strongly diminished the cytotoxic effect of fresh recipient serum against target cells. Oxisuran did not suppress the serum mediated immunological responses to the transplants significantly. With the exception that azathioprine did not significantly depress the blocking effect of heat-inactivated serum the other agents all caused a moderate depression of the cytotoxic and blocking effect of fresh and heat-inactivated recipient serum respectively.

The cellbound immunological defence as measured in the test system can be depressed in two ways; 1) The cytotoxic capacity per 10^6 lymphoid cells in the thoracic duct lymph can be depressed (table 3) and 2) the number of lymphoid cells circulating in the thoracic duct can be diminished (table 2). It is difficult to quantitate the in vivo depression of cellbound immunological defence caused by the cytopenia in the thoracic duct. Also, in the Actinomycin treated and irradiated animals there was a diminished lymph flow in the thoracic duct which further decreased the number of circulating lymphoid cells.

ALS had a most pronounced effect on cellbound immunity by depressing the number of lymphoid cells in the thoracic duct lymph to a great extent and also by strongly depressing the cytotoxic activity per 10^6 lymphoid cells. Methylprednisolone and methotrexate likewise had a marked depressive effect on cellbound immunological events in the way just mentioned. Actinomycin-C, total body irradiation and cyclophosphamide depressed the cellbound immunological events mainly by diminishing the number of lymphoid cells in thoracic duct lymph (and blood). Oxisuran suppressed the cell mediated immunological responses to the kidney transplants only by changing the cytotoxic capacity of the lymphoid cells in thoracic duct lymph. This effect was rather weak. Azathioprine appeared to be ineffective to suppress cellbound immunological responses in rats.

RELATION OF RESULTS TO IN VIVO TRANSPLANT REJECTION AND CLINICAL APPLICATIONS

The test system described here cannot exactly mimic the in vivo reaction from the immunological defence mechanisms to an allogeneic transplant. Different kinds of helper cells directed to the transplant site by chemotaxis and vascular factors are also important. However, the test system can measure the magnitude and kinetics of the attack by immunocompetent cells and antibodies with specific reactivity towards the antigens of the transplanted cells. When a rejection process is started the magnitude of this attack is most important and determines the outcome of the transplantation.

There are also differences in the allogeneic rejection process in different species and the results presented here naturally cannot directly be applied on human kidney-, liver- and heart-transplant patients. However, the tests are performed in a mammal where standardized major antigenic incompatibilities between donors and recipients can be used easily with inbred strains. The system of transplantation antigens in the rat is well known and similar to the systems of other mammals including man (Dausset, 1971; Balner & van Bekkum eds., 1971). Also the experiments presented here were performed by means of kidney transplantation. The antigens were then presented to the recipient rapidly, in large amounts and by the intravenous route that is in a way similar to that after clinical transplantation. The species differences of in vivo efficiency of immunosuppressive agents are well investigated and are usually caused by differences in the rate of metabolism of the drug (Schwartz ed., 1967). If the species peculiarities mentioned above in the discussion of the results are borne in mind, some comments valid for clinical transplantation can be made:

Antilymphocyte serum was a most potent immunosuppressive agent suppressing the most important cellbound defence to allogeneic transplants. The agent should have considerable clinical value. However, the doses per kg and day used in these experiments are by far larger than the doses used clinically. Methylprednisolone and methotrexate likewise had marked depressive effects on the cellbound immunological events. Both agents should be useful after clinical transplantation. Methotrexate treatment should be tried in combination with folinic acid which could reduce toxicity without substantial loss in immunosuppressive effect. Oxisuran having a rather weak but remarkably selective suppressive effect on the cellbound immunological responses could be of value if used

together with other immunosuppressive agents after clinical transplantations. However so far oxisuran has not been given to human beings. Actinomycin, cyclophosphamide and irradiation all depressed cellbound immunological events mainly by decreasing the number of lymphoid cells and therefore should be useful in the treatment of rejection crises already started. Azathioprine is doubtfully effective in rodents and no conclusions concerning use in other species can be drawn from these experiments.

Although total body irradiation was found most effective to depress serum-mediated cytotoxic immune responses to the transplants it is naturally not a method of choice for clinical use. It would have been of more clinical interest to investigate extracorporal blood irradiation in this test system, had it been technically feasible.

A major aim of the experiments presented here also was to find powerful immunosuppressive agents which left the blocking activity of recipient serum undisturbed. Combinations of these agents then should be suitable in immunosuppressive treatment after allogeneic transplantation. However no marked such effects were found. Only in the corticosteroid treated and cyclophosphamide treated groups there appeared to be a tendency to selective depression of the cytotoxic effect in recipient serum. A combination of these agents then could have a beneficial effect after transplantation (Starzl et al 1971) but could be less satisfactory in tumor treatment.

SUMMARY AND CONCLUSIONS

An *in vitro* test system to investigate the *in vivo* effectiveness of immunosuppressive drugs after allogeneic kidney transplantation in the rat is described. The cytotoxic effects of recipient thoracic duct lymphoid cells and fresh or heat-inactivated recipient serum against donor strain target cells were measured with ^{51}Cr release assay.

Thoracic duct lymphoid cells from kidney transplant recipient rats had a clear cytotoxic effect on donor strain kidney target cells after 12 hours of incubation. After only two hours a moderately strong cytoaggressive effect of fresh serum from recipient rats was noted. The cytotoxic effect of sensitized lymphoid cells was significantly reduced when heat-inactivated recipient serum was added to the target cells 15 min before the lymphoid cells.

The influence of presensitization, total body irradiation and several immunosuppressive drugs on the immune response evoked by allogeneic rat kidneys was studied. It was found that the procedures influenced the isotope release in the test system in different ways.

It was concluded that:

Rats that had undergone two consecutive allogeneic transplantations from isogenous donors had thoracic duct lymphoid cells and serum with an increased cytotoxic effect against donor strain target cells as compared to control rats transplanted only once. Heat-inactivated recipient serum from such animals also had an increased blocking effect on cell-mediated cytotoxicity.

ALS had a most pronounced depressive effect on the cell-bound immunological responses evoked by the transplants in the recipient rats, decreasing both the number of lymphoid cells in the thoracic duct lymph and the cytotoxic activity of the remaining cells. Methylprednisolone and methotrexate also strongly suppressed the cellular immunological events in the way just mentioned. Actinomycin-C, cyclophosphamide and total body irradiation depressed cell-bound immune response mainly by causing a decrease in the number of cells in the thoracic duct lymph. Oxisuran suppressed the cellbound immune response only by decreasing the cytotoxic activity of the thoracic duct lymph cell population. Azathioprine did not influence cell-bound immunological responses to allogeneic kidney transplants.

Total body irradiation had a superior depressive effect on cytotoxic and blocking activity in recipient serum as compared to the immunosuppressive drugs investigated in this communication ($p < 0.01$): Methotrexate likewise had a marked depressive effect on antibody mediated immunological responses. Cyclophosphamide and methylprednisolone treatment strongly diminished the cytotoxic effect of fresh recipient serum against target cells. Oxisuran did not suppress the serum mediated immunological responses to the transplants. With the exception that azathioprine did not significantly depress the blocking effect of heat-inactivated recipient serum the other agents (azathioprine, ALS, actinomycin-C) all caused a moderate depression of the cytotoxic and blocking effect of fresh and heat-inactivated recipient serum, respectively.

When folinic acid was given regularly between consecutive methotrexate injections, the depression of the lymphoid cell amount in the thoracic duct lymph was markedly less. The results of the cytotoxicity tests were similar to those obtained with methotrexate treatment alone.

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Miss Karin Larsen for instruction in microsurgical kidney transplantation technique.

Dr H. H. Freedman for supplying Oxisuran.

FIGURES

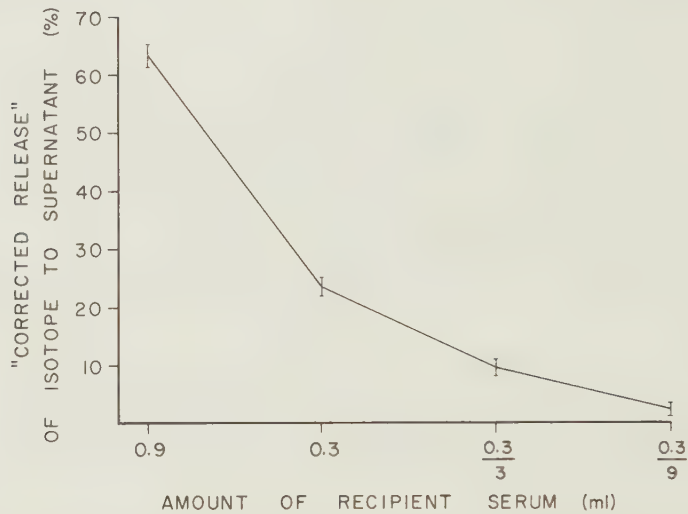


Fig 1 Per cent corrected release of ^{51}Cr to supernatant from 10^5 WF target cells exposed to different amounts of BN recipient serum and a constant amount of complement for 2 hours. Mean $\pm$ SD from 4 experiments. 70-75 % uncorrected isotope release (68-73 % corrected release) represents 100 % target cell death.

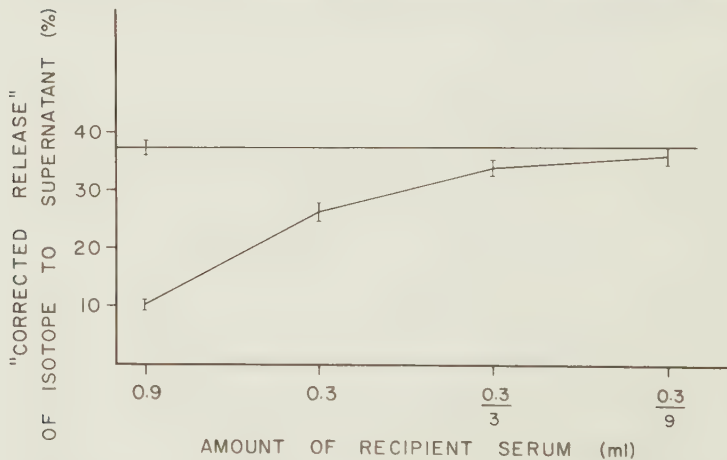


Fig 2 Per cent corrected release of ^{51}Cr to supernatant from 10^5 WF target cells exposed to 10^6 BN recipient thoracic duct lymphoid cells and different amounts of heat-inactivated recipient serum for 12 hours. The horizontal line shows the isotope release with target cells and lymphoid cells alone. Mean $\pm$ SD from 4 experiments.

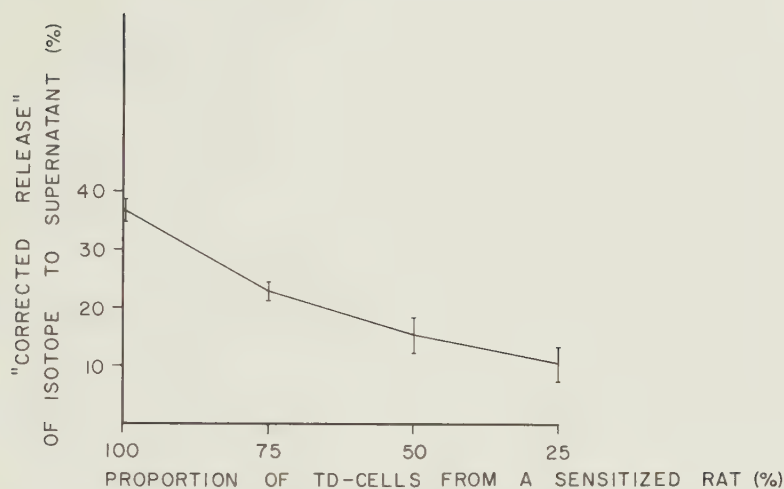


Fig 3 Per cent corrected release of ^{51}Cr to supernatant from 10^5 WF target cells exposed to 10^6 BN thoracic duct lymphoid cells containing 100, 75, 50 or 25 per cent immune cells and 0, 25, 50 or 75 per cent normal lymphoid cells for 12 hours. Mean $\pm$ SD from 4 experiments.

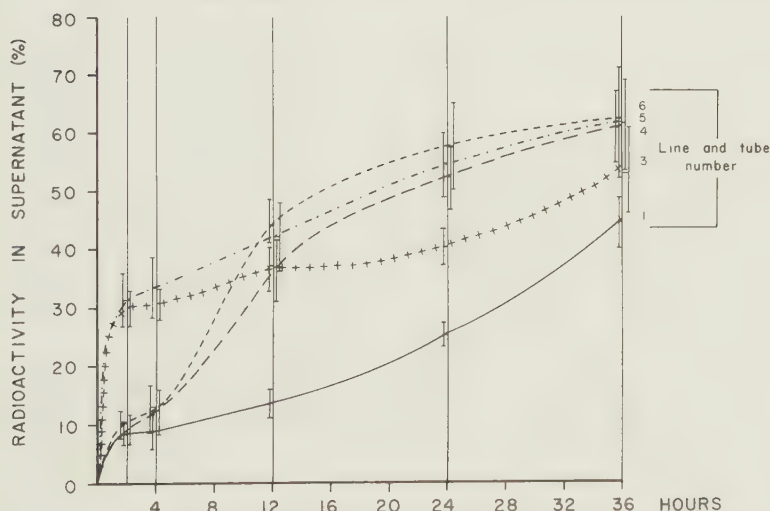


Fig 4 Release of ^{51}Cr to supernatant in non-immunosuppressed group, precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers (= tube numbers in Table 1): 1) WF kidney cells only; 3) WF kidney cells + complement containing BN recipient serum; 4) WF kidney cells + BN recipient lymphoid cells + heat-inactivated BN recipient serum; 5) WF kidney cells + BN recipient lymphoid cells + complement containing BN recipient serum; 6) WF kidney cells + BN recipient lymphoid cells.

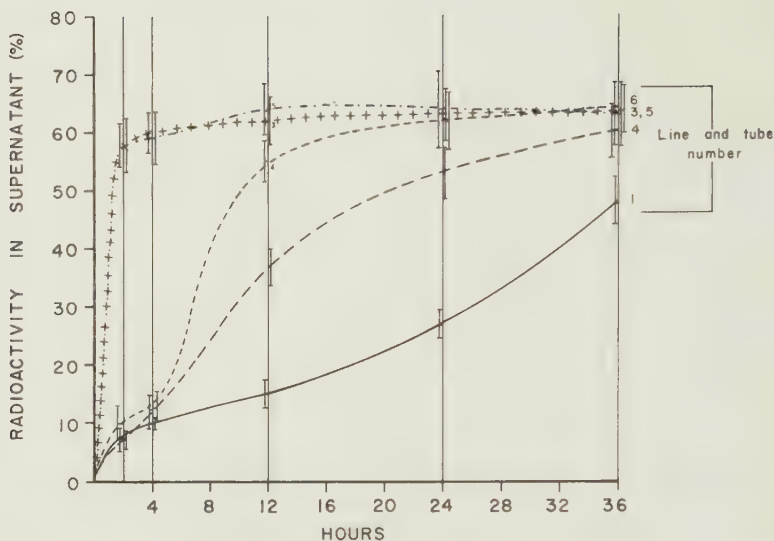


Fig 5 Release of ^{51}Cr to supernatant in the presensitized group; (Group II). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

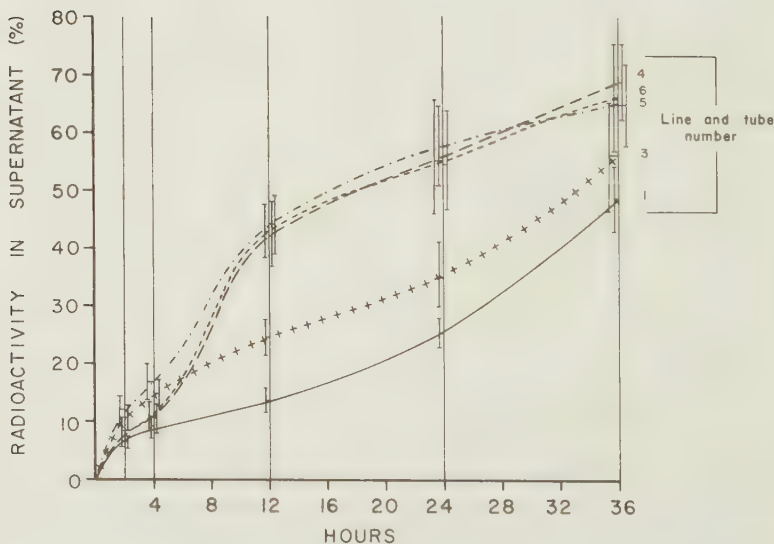


Fig 6 Release of ^{51}Cr to supernatant in the irradiated group (Group III). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

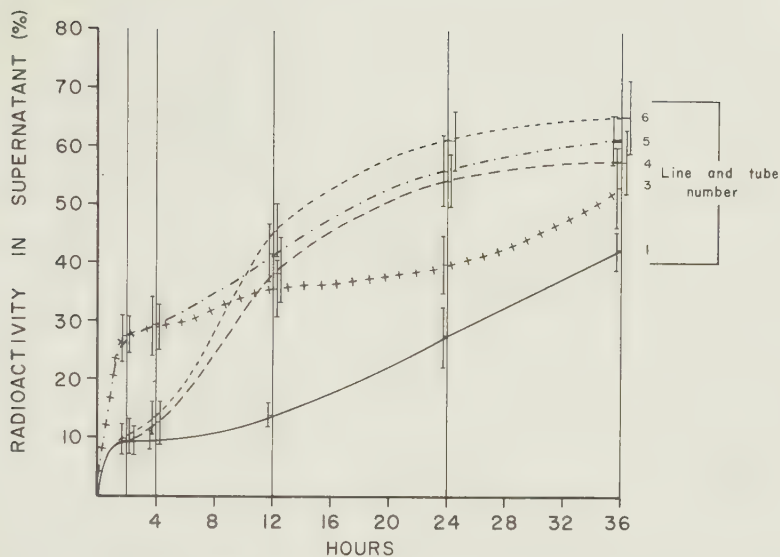


Fig 7 Release of ^{51}Cr to supernatant in the azathioprine treated group (Group IV). Precise values only known at the vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

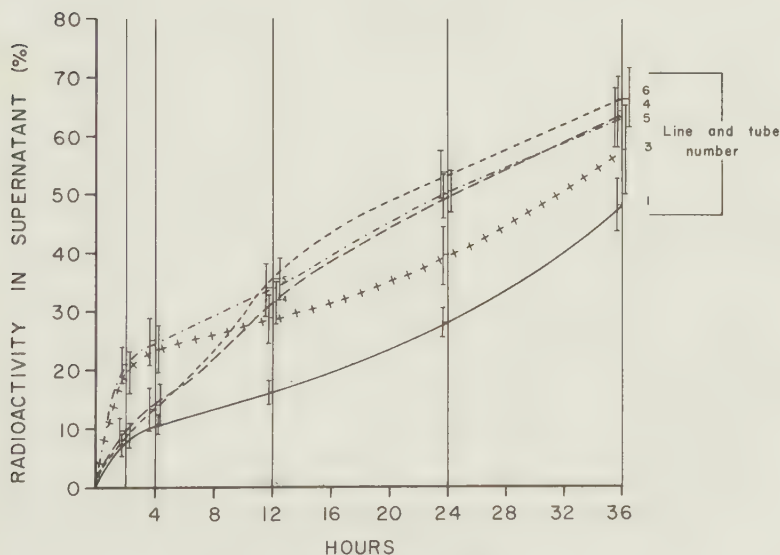


Fig 8 Release of ^{51}Cr to supernatant in the methylprednisolone treated group (Group V). Precise values only known at the vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

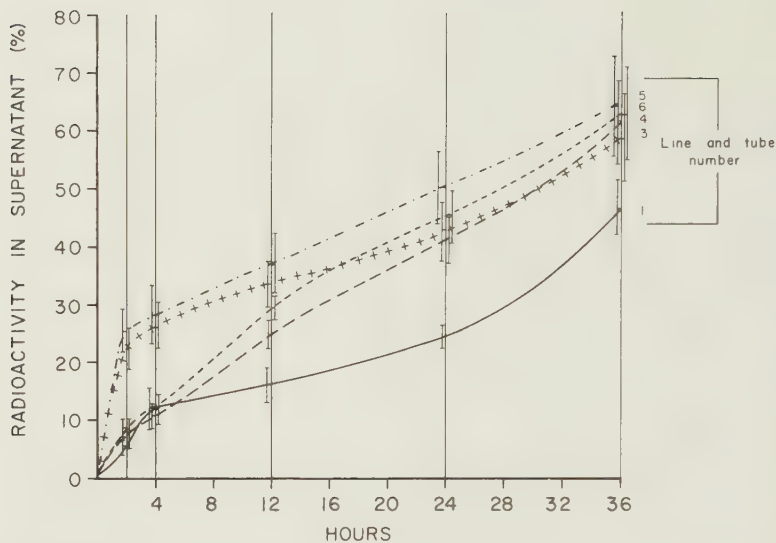


Fig 9 Release of ^{51}Cr to supernatant in the ALS treated group (Group VI). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

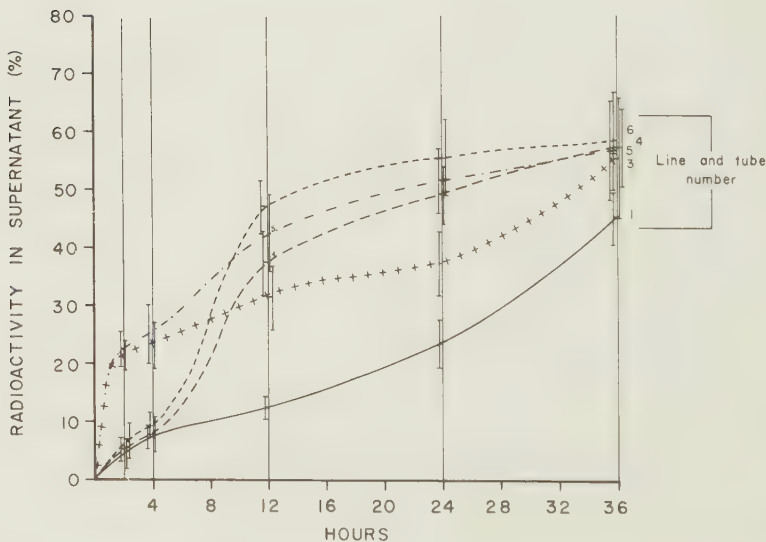


Fig 10 Release of ^{51}Cr to supernatant in the actinomycin treated group (Group VII). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

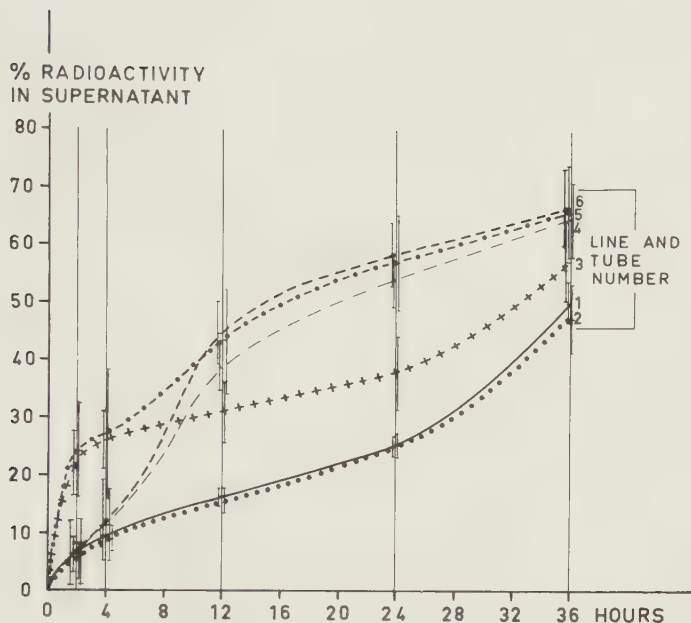


Fig 11 Release of ^{51}Cr to supernatant in the cyclophosphamide treated group (Group VIII). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

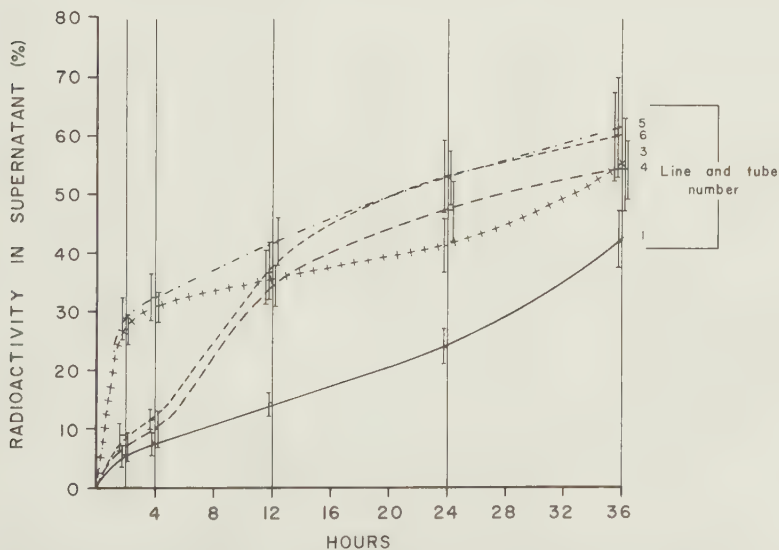


Fig 12 Release of ^{51}Cr to supernatant in the oxisuran treated group (Group IX). Precise values only known at the vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

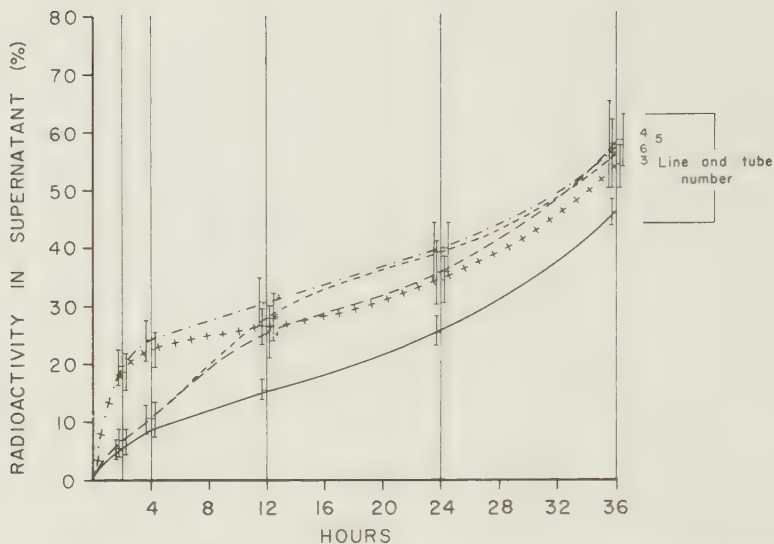


Fig 13 Release of ^{51}Cr to supernatant in the methotrexate treated group (Group X) Precise values only known at the vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

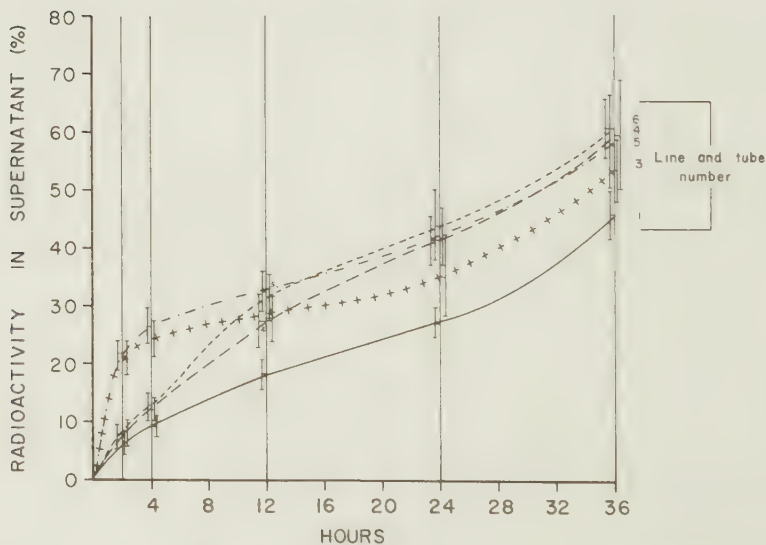


Fig 14 Release of ^{51}Cr to supernatant in the methotrexate-folinic acid treated group (Group XI). Precise values only known at vertical lines (mean $\pm$ SD). Not "corrected release". Line numbers as in fig 4.

Table 1. Thoracic duct cells and/or serum samples that were added to 10^{5-6} ^{51}Cr labelled WF kidney cells in different test tubes. The number of viable thoracic duct lymphoid cells (TD-cells) were always 10^6 . Serum was fresh when not indicated as heat-inactivated (i. a.).

		Tube number*
Control tubes	0.3 ml WF normal i. a. serum	1
	BN normal TD-cells + 0.3 ml BN normal i. a. serum	2 **
Tubes with test lymphoid cells and/or serum	0.3 ml BN recipient serum + 0.1 ml BN normal serum	3
	BN recipient TD-cells + 0.3 ml BN recipient i. a. serum	4
	BN recipient TD-cells + 0.3 ml BN recipient serum	5
	BN recipient TD-cells + 0.3 ml BN normal i. a. serum	6
Complement control tubes	0.3 ml BN recipient serum + 0.5 ml BN normal serum	7 **
	BN recipient TD-cells + 0.4 ml BN normal serum	8 **

* all sera added 15 min before lymphoid cells

** performed in the experiments from 5 animals in each experimental group

Table 2. Number of white blood cells (WBC) and lymphocytes per mm³ in peripheral blood and thoracic duct lymph in recipient rats belonging to different experimental groups.

	Blood		Lymph
	Pre transpl.	5 d. post transpl.	14 d. post transpl.
Non-immunosuppressed group (group I)			
WBC	15400 ± 3300	20600 ± 5500	36200 ± 3800
Lymphocytes	12100 ± 2900	11200 ± 4900	35500 ± 3700
Presensitized group (group II)			
WBC	21500 ± 4200	23100 ± 5400	35000 ± 3000
Lymphocytes	11400 ± 2800	12100 ± 3600	33800 ± 2700
t value WBC group I/II	3.15*	0.98	0.73
t value lymphocytes group I/II	0.49	0.44	0.94
Irradiated group (group III)			
WBC	18200 ± 4100	14600 ± 9500	7400 ± 2500
Lymphocytes	13300 ± 3400	7400 ± 5000	7100 ± 2400
t value WBC group I/III	1.60	1.64	18.72***
t value lymphocytes group I/III	0.83	2.30*	19.07***
Azathioprine treated group (group IV)			
WBC	19500 ± 6000	30300 ± 5800	35400 ± 3700
Lymphocytes	14300 ± 6300	19200 ± 6400	34000 ± 3600
t value WBC group I/IV	1.81*	3.37**	0.43
t value lymphocytes group I/IV	-	2.97**	0.73
Methylprednisolone treated group (group V)			
WBC	18300 ± 3100	23500 ± 6500	24600 ± 2400
Lymphocytes	14000 ± 2600	14400 ± 4200	23800 ± 2200
t value WBC group I/V	1.93*	1.00	7.75***
t value lymphocytes group I/V	1.51	1.50	8.04***

ALS treated group (group VI)				
WBC				8600 ± 2900
Lymphocytes				8400 ± 2900
t value WBC group I/VI	16200 ± 3000	11300 ± 3500	13300 ± 4000	17.09***
t value lymphocytes group I/VI	12400 ± 2300	5300 ± 2200	7100 ± 3600	17.27***
	0.55	* 4.28***	3.34**	
	0.31	3.30**	3.64***	
Actinomycin treated group (group VII)				
WBC	18100 ± 2400	25100 ± 8700	7500 ± 6100	15100 ± 2600
Lymphocytes	13400 ± 2400	15200 ± 7400	3800 ± 2900	14700 ± 2600
t value WBC group I/VII	1.99*	1.30	4.90***	13.60***
t value lymphocytes group I/VII	1.07	1.36	5.61***	13.70***
Cyclophosphamide treated group † (group VIII)				
WBC	10100 ± 2700	4900 ± 1700	4200 ± 1700	22600 ± 2500
Lymphocytes	7800 ± 2700	3000 ± 1500	2000 ± 800	21500 ± 2400
t value WBC group I/VIII	0.36	11.57***	8.32***	10.61***
t value lymphocytes group I/VIII	0.45	4.44***	4.18***	10.97***
Oxisuran treated group (group IX)				
WBC	17400 ± 3700	20400 ± 6500	22400 ± 7000	33500 ± 5800
Lymphocytes	13900 ± 2700	13900 ± 4800	14300 ± 6000	32500 ± 5500
t value WBC group I/IX	1.22	0.11	0.46	1.15
t value lymphocytes group I/IX	1.40	1.20	0.02	1.26
Methotrexate treated group (group X)				
WBC	15100 ± 2100	8900 ± 6400	8000 ± 5100	11500 ± 3200
Lymphocytes	11900 ± 1900	7000 ± 6900	5700 ± 4400	10900 ± 3600
t value WBC groups I/X	0.17	4.16***	5.16***	14.80***
t value lymphocytes groups I/X	0.51	1.90*	4.00***	14.18***
Methotrexate-Folinic acid treated group (group XI)				
WBC	16800 ± 5800	12500 ± 5100	9900 ± 4300	18900 ± 2400
Lymphocytes	13200 ± 4500	8500 ± 3200	6800 ± 2900	18200 ± 2200
t value WBC group I/XI	0.63	3.29**	4.73***	11.41***
t value lymphocytes group I/XI	0.61	1.41	4.05***	11.84***

Homogenous variance; DF = 18; $p < 0.001^{***}$, $t > 3.610$; $p < 0.01^{**}$, $t > 2.552$ (significant); $p < 0.05^*$, $t > 1.734$

† Compared to an earlier control group (Husberg, 1972).

Table 3. Per cent release of ^{51}Cr to supernatant in the different experimental groups. "Corrected release".

Tube and line number

	3				4			
	Target cells + serum				Target cells + lymphoid			
	(fresh)				cells + serum (heatinact)			
	2hr	4hr	12hr	24hr	2hr	4hr	12hr	24hr
Non-immunosuppressed group								
(group I)								
Mean	23.0	23.5	26.6	20.5	1.5	3.5	27.3	36.3
SD	2.19	1.75	2.78	2.97	-	-	2.22	4.23
Presensitized group								
(group II)								
Mean	54.3	54.9	55.4	49.1	0.2	0.7	26.3	35.3
SD	3.24	2.83	2.65	2.90	-	-	2.46	2.67
t value group I/II	23.90***	28.28***	22.44***	20.62***	-	-	0.91	0.61
Irradiated group								
(group III)								
Mean	5.0	7.4	12.6	13.5	1.8	3.4	33.1	41.3
SD	1.52	1.86	1.64	3.94	-	-	3.7	5.47
t value group I/III	20.23***	19.03***	12.97***	4.23***	-	-	-	-
Azathioprine treated group								
(group IV)								
Mean	18.5	20.9	25.9	16.9	0.8	3.5	29.0	37.4
SD	3.72	3.17	2.98	4.59	-	-	4.14	3.05
t value group I/IV	3.15**	2.18*	0.52	1.97*	-	-	-	-
Methylprednisolone treated group (group V)								
Mean	13.5	14.7	14.7	15.0	2.6	3.0	15.4	28.8
SD	3.00	3.68	3.97	5.56	-	-	3.69	2.90
t value group I/V	7.76***	6.50***	7.37***	2.64**	-	-	-	-
ALS treated group								
(group VI)								
Mean	18.5	19.7	21.1	24.6	2.1	0.4	10.7	23.4
SD	1.58	2.68	1.98	2.45	-	-	1.63	3.43
t value group I/VI	5.17***	5.44***	4.80***	3.21**	-	-	-	-
Actinomycin treated group								
(group VII)								
Mean	18.0	16.9	21.2	17.8	1.0	1.2	29.0	37.7
SD	2.61	2.32	4.88	4.23	-	-	5.07	3.29
t value group I/VII	4.32***	6.88***	2.45*	1.61	-	-	-	-
Cyclophosphamide treated group								
(Group VIII) * †								
Mean	15.8	18.4	19.4	16.8	0.9	1.8	27.4	38.0
SD	2.69	2.08	2.11	1.56	-	-	1.70	1.33
t value group I/VIII	7.00***	7.57***	6.03***	8.23***	-	-	-	-
Oxixuran treated group								
(Group IX)								
Mean	23.1	25.2	25.3	23.4	1.8	2.9	15.3	31.4
SD	1.77	1.56	3.16	3.46	-	-	1.12	3.59
t value group I/IX	0.04	2.18*	0.88	1.86*	-	-	-	-
Methotrexate treated group								
(group X)								
Mean	14.1	14.9	13.2	10.9	1.2	2.4	12.0	17.0
SD	2.22	2.15	2.65	3.71	-	-	3.77	5.22
t value group I/X	8.66***	9.42***	10.42***	6.05***	-	-	-	-
Methotrexate-Folinic acid treated group (group XI)								
Mean	15.6	16.6	13.9	10.3	1.5	3.2	13.1	19.1
SD	2.00	2.48	3.92	5.65	-	-	3.18	3.81
t value group I/XI	7.58***	6.95***	7.95***	4.80***	-	-	-	-

Homogenous variance; DF = 18; $p < 0.001$ ***, $t > 3.610$; $p < 0.01$ **, $t > 2.552$ (significant); $p < 0.05$, $t > 1.734$;

* See text. Materials and Methods

†† Compared to an earlier control group (Husberg, 1972)

5 Target cells + lymphoid cells + serum (fresh)				6 Target cells + lymphoid cells				Target cells + group I lymphoid cells +group I-XI serum(heatinact) †
2hr	4hr	12hr	24hr	2hr	4hr	12hr	24hr	12hr
24.1	26.3	33.6	38.7	1.5	4.0	36.2	43.0	26.5
2.29	2.35	2.98	4.45	-	-	1.76	4.63	1.52
55.6	54.3	58.2	50.4	2.2	3.7	47.2	47.0	15.0
3.36	3.38	3.37	5.09	-	-	2.58	3.30	1.11
23.24	***20.37	***16.40	5.18	***-	-	10.58	***2.11*	10.58***
5.7	9.0	35.1	41.4	1.7	3.1	35.2	40.6	35.5
1.78	1.84	3.90	5.77	-	-	3.12	7.78	1.57
19.47	***17.59	***0.95	1.04	-	-	0.89	0.76	7.10***
20.9	21.7	32.1	39.4	1.1	4.4	36.4	46.7	29.1
3.64	4.38	2.40	4.74	-	-	3.33	3.66	1.32
2.12*	2.77**	1.15	0.32	-	-	0.17	1.91*	2.21*
15.1	15.9	21.1	30.6	2.1	3.7	23.4	34.1	29.7
2.22	3.58	3.80	2.98	-	-	2.21	3.06	1.10
8.52	***7.32	***7.81	4.57	***-	-	13.82	***4.82	2.91**
20.9	20.9	24.3	33.8	3.3	0.5	15.2	28.2	30.1
3.72	4.02	2.92	6.65	-	-	1.82	3.04	1.30
2.23*	3.49**	6.64***	1.94*	-	-	24.10***	7.95***	3.06**
19.0	19.5	34.5	36.9	2.7	2.6	40.5	40.5	30.4
2.40	3.39	5.35	4.70	-	-	3.57	4.38	1.29
4.56	***4.91	***0.47	0.83	-	-	-	1.19	3.35**
17.0	19.7	33.1	42.6	2.3	3.1	35.5	44.0	-
7.31	7.65	6.10	5.90	-	-	2.16	2.19	-
3.06**	2.73**	0.01	0.12	-	-	2.62**	3.42**	-
25.0	27.4	32.4	39.1	3.1	5.0	27.6	39.3	27.2
2.97	2.68	2.06	3.44	-	-	2.73	5.16	1.27
1.05	0.91	0.96	0.21	-	-	7.98	1.60	0.61
14.7	16.7	17.8	18.0	2.5	2.6	15.0	19.7	31.5
3.03	2.20	2.50	3.43	-	-	3.26	4.83	1.49
7.42	***9.10	***12.24	11.05	***-	-	17.21	***10.50	4.00***
16.0	19.1	19.1	19.3	2.4	4.0	17.5	22.5	29.5
1.87	1.67	2.00	3.95	-	-	2.77	4.15	0.97
8.13	***7.61	***12.15	9.80	***-	-	17.20	***9.91	2.83***

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